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Expression of apoptosis regulatory proteins in tubular epithelium stressed in culture or following acute renal failure

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Background. While tubular cell death is a characteristic of acute renal failure (ARF), the molecular mechanisms that modulate this cell death are unclear. Cell fate in acute renal failure hinges on a balance of survival and mortality factors in a changing environment. We further explored this issue by studying selected cell death-related proteins in experimental renal failure.

Method. The expression of genes that promote (c-*myc*, *Bax*, *BclxS*) or protect (*Bcl2*, *BclxL*) from cell death was studied by Northern blot, Western blot, and immunohistochemistry in murine kidneys following ARF induced by folic acid or in renal tubular epithelial cells (MCT) stressed in culture.

Results. Renal mRNA levels encoding for *c-myc* and *BclxL* were elevated in ARF while the *Bcl2*/*Bax* ratio was decreased (*Bcl2* decreased and *Bax* increased; $P < 0.05$). Protein levels of BclxL increased and Bcl2 protein decreased. Expression of tumor necrosis factor (TNF-α), a mediator of ARF, was also increased. Immunohistochemistry further demonstrated that BclxL was increased in some tubuli and absent in others, while Bcl2 expression decreased diffusely. Bax staining was also patchy among tubuli and individual cells in the tubular wall and lumen. As a relative deficit of survival factors is present in ARF, MCT epithelium were deprived of serum survival factors. This resulted in apoptosis, decreased *Bcl2*/*Bax* and *BclxL*/*Bax* ratios ($P < 0.05$) and sensitization to TNF-α-induced apoptosis ($P < 0.05$). The latter was prevented by enforced overexpression of *BclxL* ($P < 0.01$). TNF-α increased the mRNA levels encoding for *c-myc* and decreased *BclxL* expression. Neither MCT cells nor the kidney expressed *BclxS*.

Conclusions. A relative deficit of survival factors likely contributes to changes in levels of BclxL and Bax in ARF. These deficits predispose to cell death induced by persistent lethal factors such as TNF-α that is increased in ARF and a potential source of increased c-myc, a downstream facilitator of cell death. These findings implicate members of the Bcl2 family of proteins as regulators of tubular cell death in ARF and single them out as potential therapeutic targets.

Key words: apoptosis, Bcl2, Bax, BclxL, acute renal failure, kidney, tubular cells, TNF.

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Acute renal failure (ARF) is often associated with renal tubular cell death. In experimental models of ARF cell death is partly mediated by apoptosis [1]. Apoptotic cells have also been demonstrated in human kidneys with ARF [2]. In addition, apoptotic tubular cell death may facilitate the tubular atrophy of chronic renal injury [3]. At present, however, there is little information on the factors that promote or regulate apoptosis in kidney.

The balance between factors that contribute to survival growth or lethality often impacts on the chance of cell death [4, 5]. For example, competition for limiting amounts of survival factors results in the apoptotic loss of 50% of newly formed oligodendrocytes during central nervous system development [6]. A similar balance may play a role in determining the extent of cell death in ARF. In fact, there is evidence that a relative deficit of survival factors contributes to ARF. Multifunctional cytokines such as insulin-like growth factor (IGF-1) and endothelial growth factor (EGF) have survival factor activity for tubular epithelium. The local expression of cytokines with survival factor activity is decreased in ARF [7–10] and the cells compete for these cytokines by increasing the expression of receptors [7, 10]. The exogenous administration of cytokines with survival factor activity improves the evolution of ARF and reduces the amount of apoptosis [11, 12]. However, the molecular mode of action of survival factors is not well understood.

Other multifunctional cytokines such as tumor necrosis factor-α (TNF-α) also promote apoptosis and induce acute tubular necrosis [13, 14], while anti-TNF-α antibodies protect against renal failure in model systems [15]. Although not conclusive, these findings are consistent with the hypothesis that changes in the balance between factors with survival and mordant activity in

tubular epithelium may modulate cell death genes in ARF, and thus determine cell fate.

Some apoptosis regulatory proteins relevant to renal pathology have been characterized. These include members of the Bcl2 family of proteins (Bcl2, Bax, Bclx, and others) and the transcription factor c-myc [16–19]. The main known function of Bcl2 family members is to regulate cell death. Bcl2 is an intracellular membrane-associated protein whose expression prevents or delays apoptotic cell death in response to a number of stimuli, including deprivation of survival factors and, in some cells, activation of TNF-α receptors [19]. Bcl2 also protects against cell death where there are morphological features of necrosis such as death induced by buthionine sulfoximine in neural cells [20]. In this regard the same stimulus, depending on the intensity, may induce either apoptosis or necrosis. The protective effect of Bcl2 is also evident in a model of ischemic cell death in which both modes of cell death have been identified: Bcl2 overexpression in the brains of transgenic mice decreased experimental infarct size 50% [21].

Bclx, a member of the Bcl2 family, has two alternatively spliced forms: BclxL and BclxS. BclxL, like Bcl2, protects cells from a wide variety of apoptotic stimuli [16]. In contrast, BclxS antagonizes the protective effect of Bcl2 and BclxL [16]. Bax is a Bcl2-like protein that binds to and antagonizes the protective effect of Bcl2 and BclxL, rendering cells more sensitive to death [18]. In this sense, the ratio of expression of Bcl2 or BclxL to Bax or BclxS appears to determine cell fate in an adverse microenvironment [18].

Finally, c-myc favors cell death when the microenvironment is adverse for survival. Overexpression of c-myc can make cells more dependent on external growth factors for the prevention of apoptosis [17]. This growth factor dependence can be overcome by overexpression of protective proteins such as Bcl2 [22].

To better understand the determinants of renal cell death, we examined the expression of the aforementioned genes under two conditions. In vivo, we examined their expression in a model of murine ARF. In vitro, we examined their expression in cultured tubular epithelium which were forced into an apoptotic program by serum deprivation or by TNF-α stimulation.

METHODS

Animals and cell culture

SJL mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). Folic acid nephropathy was induced in 5- to 7-week-old mice by a single intraperitoneal injection of folic acid (Sigma, St. Louis, MO, USA) 250 mg/kg in 0.3 mol/L sodium bicarbonate [23]. Three mice were killed at different time points (from 6 h to 7 days) after injection of either folic acid or vehicle (0.3 mol/L sodium bicarbonate). Six additional mice were killed at 24 hours. The kidneys were saline perfused. One kidney from each mouse was fixed in buffered formalin, included in paraffin and stained with hematoxylin-eosin or used for immunohistochemistry. The other was snap-frozen in liquid nitrogen for RNA, DNA and protein studies. Blood was drawn from the heart and serum creatinine was determined at 24 hours.

MCT cells are a cultured line of proximal tubular cells harvested originally from the renal cortex of SJL mice [24]. The cells were maintained in culture in DMEM supplemented with penicillin 100 μ/mL and streptomycin 100 μg/mL and 10% heat-inactivated fetal calf serum (FCS; DMEM-10%) as previously described [24]. A human BclxL cDNA (a generous gift from Craig B. Thompson, University of Chicago) was cloned into a vector which contained a 1.2 kb fragment of rat gamma glutamyl transferase promoter and the human GH polyadenylation signal. This vector directs expression in tubular cells [25]. MCT cell lines constitutively expressing human BclxL or an empty control vector were established. For that purpose MCT cells were electroporated at 960 μF and 220 V and cultured in the presence of 600 μg/mL G418 for three weeks. An increased expression of BclxL was confirmed in MCT-BclxL by Western blot.

For those experiments in which the effect of serum deprivation or TNF-α stimulation was examined, the cells were plated and then grown for 24 hours in DMEM-10%. Then the media was replaced with fresh DMEM-10% or serum-free DMEM (DMEM-0%). The DMEM-0% contained the same supplements as the serum-supplemented DMEM with the exception of the FCS.

Murine TNF-α (60 U/ng; Boehringer Mannheim GmbH, Mannheim, Germany) was added to some cultures to achieve a final concentration of 30 ng/mL. When TNF-α was added to serum-free cultures, the cultures were grown for 24 hours in DMEM-0% prior to the addition of the TNF-α. Preliminary experiments had demonstrated that this concentration of TNF-α is cytotoxic.

Assessment of cell death/apoptosis

For quantification of cell death, 10,000 cells were seeded in 24-well plates (Corning Costar, Cambridge, MA, USA) in DMEM-10%, and after 24 hours the culture conditions were modified as described above. Thereafter at defined time points, the cells were harvested from the wells. The nonadherent cells were pooled with the adherent cells, which were detached from the plate by gentle trypsinization. The total number of cells were counted and the cell viability was determined by trypan blue exclusion. For this purpose at least 100 cells from triplicate wells were counted in a Fuch-Rosenthal chamber.

Apoptosis was also assessed by flow cytometry [26, 27]. Cells attached to the plate were collected and mixed

with detached cells present in the supernatant. Cells were spun and resuspended in 100 μg/mL propidium iodide, 10 μg/mL RNAse A, 0.05% NP-40 in phosphate-buffered saline (PBS), incubated at 4°C for one hour, and analyzed on the FACScan using LYSIS II software. The percentage of hypodiploid cells, as assessed by decreased DNA staining (A_0), comprising apoptotic cells with fragmented nuclei, was counted. For comparisons between cells overexpressing BclxL and control cells, the percentage of specific cell death was quantified. For that purpose, cell death in the absence of TNF-α was subtracted from cell death in the presence of TNF-α. Cell death thus calculated in control cells was considered to be 100%.

To assess for the pyknotic nuclear changes seen in apoptosis, cells were stained with propidium iodide. MCT cells were plated onto Labtek™ slides (Nunc Inc., Napersville, IL, USA) in DMEM-10%. After 24 hours the media was changed to either fresh DMEM-10% or DMEM-0% and then grown for an additional 48 hours. The cells were stained with propidium iodide basically as previously described [28]. Briefly, the slides were washed with PBS, fixed for 10 minutes in 10% buffered formalin, washed with PBS, stained for 30 minutes at 37°C in 0.1 μg/mL propidium iodide, 100 μg/mL RNAse A, in PBS pH 7.2 and mounted in 90% glycerol solution. The slides were examined with a Zeiss microscope (Carl Zeiss Inc., Thornwood, NJ, USA) using an ultraviolet light source filtered for propidium iodide. Images were photographed on Kodak TMAX 3200 film and printed at equivalent exposures.

Internucleosomal DNA fragmentation of genomic DNA, a characteristic of apoptosis, was assessed both in whole kidney and in MCT cells. For this purpose, kidney samples or 10^6 cultured cells were lysed in 400 μL of hypotonic lysis buffer (100 mmol/L NaCl, 100 mmol/L Tris, 1 mmol/L EDTA, 1% SDS in PBS, pH 7.2) with 200 μg/mL proteinase K overnight at 37°C. DNA was precipitated, resuspended and separated in a 1.5% agarose gel. The DNA fragmentation ladder was demonstrated with ethidium bromide staining of the gel and/or Southern blotting. For the Southern blotting, the DNA was transferred onto nylon membranes (Genescreen; NEN, Boston, MA, USA) and probed with MCT genomic DNA which was radiolabeled with [^{32}P]CTP by random priming (Boehringer-Mannheim, Indianapolis, IN, USA) [13]. Similar results were obtained when the DNA was prepared from 2×10^6 cells which were lysed in 400 μL 0.2% Triton X-100, 10 mmol/L Tris, 1 mmol/L EDTA pH 8. The lysate was then cleared by centrifugation for 15 minutes at 13,800 *g*. The DNA in the clarified supernatant was precipitated and then processed as described above.

Northern hybridization

For Northern blotting, 30 μg of total RNA was separated in 1% agarose gels containing 2.3% formaldehyde [29]. RNA was transferred to nylon membranes (Genescreen Plus; NEN) and pre-hybridized and hybridized at 65°C in 6× SSC, 5× Denhardts, 10% dextran sulfate, 1% SDS, 100 μg/mL salmon sperm DNA and 100 μg/mL polyadenylic acid. The probes were labeled by random priming and added to the hybridization solution to a final activity of 1.5×10^6 cpm/ml. After hybridization, the blots were washed twice in 2× SSPE for 15 minutes at room temperature, once in 2× SSPE, 2% SDS for 45 minutes at 65°C, and once in 0.1× SSPE, 0.1% SDS for 15 minutes at 65°C. The blots were then exposed to film at −70°C with the use of an intensifying screen. Blots were stripped and subsequently rehybridized with the probe for 18S or the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to account for small loading or transfer variations. For quantitative purposes, the autoradiograph were scanned with a densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA).

Probes for murine Bcl2, murine Bax, murine bclx and human BclxL have already been described [29]. The murine probes were prepared by PCR of reverse transcribed total RNA isolated from MCT cells. They were cloned and sequenced. The human BclxL probe is specific for the BclxL transcript of the *bclx* gene, and it has also been cloned and sequenced. The c-myc probe was a gift from Dr. William Lee, Department of Medicine, University of Pennsylvania.

Western blot

Tissue or cell samples were homogenized in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% Triton X-100, 0.3% NP-40, 0.1 mmol/L PMSF and 1 μg/ml pepstatin A) then separated by 12% SDS-PAGE under reducing conditions. After electrophoresis, samples were transferred to PVDF membranes (Millipore Corp., Bedford, MA, USA), blocked with 5% skimmed milk in PBS/0.5% vol/vol Tween 20 for one hour, washed with PBS/Tween, and incubated with the following antibodies: rabbit polyclonal anti-$Bclx_{S/L}$ antibodies (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Bax antibodies (1:2000; Pharmingen, San Diego, CA, USA), rabbit anti-Bcl2 antibodies (1:2000; Pharmingen), and anti-TNF-α antibodies (1:200; Santa Cruz Biotechnology). All antibodies were diluted in 5% milk PBS/Tween. Blots were washed with PBS/Tween and subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000; Amersham, Aylesbury, UK). After washing with PBS/Tween, the blots were developed with the enhanced chemiluminescence method (ECL) following the manufacturer's instructions (Amersham).

Immunohistochemistry

Paraffin-embedded tissue sections, 5 μm thick, were floated onto APES (Sigma) coated slides. Slides were

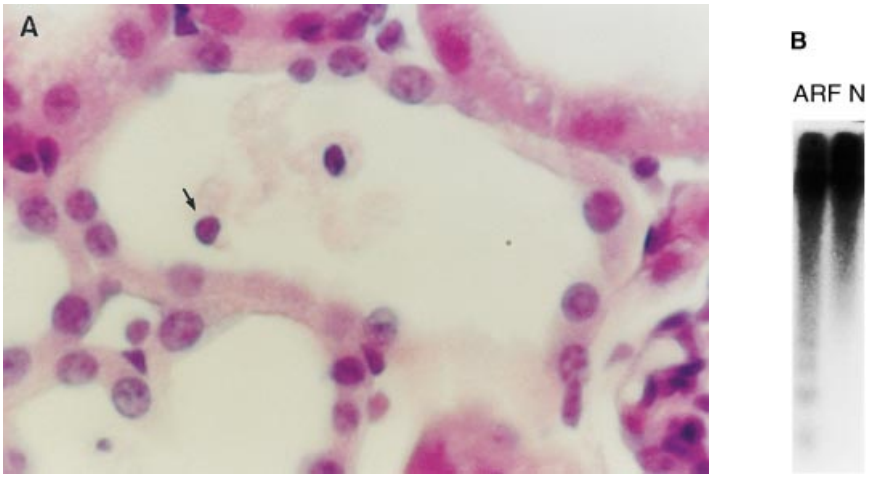

Fig. 1. Apoptosis in acute renal failure (ARF). (*A*) Apoptotic cells were noted in tubuli from mice with ARF after 24 hours of folic acid injection. Note apoptotic cells with condensed chromatin in the tubular lumen (H & E, original magnification ×1000). No such cells were observed in normal kidneys. (*B*) Internucleosomal DNA degradation was present at 24 hours in kidneys from mice with ARF, but not in normal kidney (N).

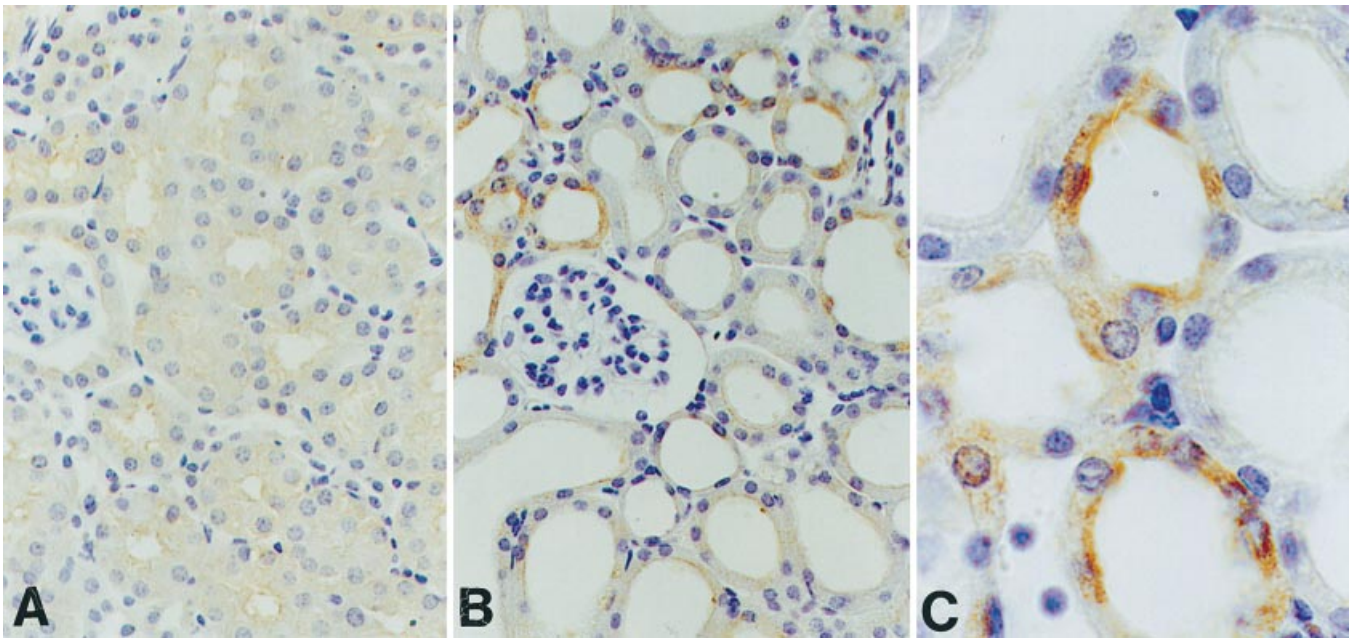

Fig. 3. Immunohistochemistry of kidneys after 24 hours of the injection of folic acid or vehicle. (*A–C*) BclxL. Tubuli with both increased and decreased staining are present in ARF (*D*, *E*) Bcl2. A diffuse decrement in Bcl2 is noted in ARF. (*F*, *G*) Bax. Note detached tubular cells stained with anti-Bax in G. (*H*, *I*) Negative control stained in which nonspecific IgG was used as primary antibody. (A, D, F, H) Control kidneys; (B, C, E, G, I) ARF kidney, (original magnification ×400, except C, ×1000).

deparaffined with xylene and dehydrated in graded concentrations of ethanol. Endogenous peroxidase was quenched with 3% H_2O_2:methanol (1:1) for 30 minutes at room temperature. Sections were rinsed twice in PBS and then blocked with 6% horse serum and 4% BSA in PBS for one hour at room temperature. Primary antibodies were: rabbit polyclonal anti-$Bclx_{S/L}$ (1:100; Santa Cruz Biotechnology), rabbit anti-Bax serum (1:1000; Pharmingen), rabbit anti-Bcl2 serum (1:200; Pharmingen). They were diluted in 1% horse serum and 4% BSA in PBS and left overnight at 4°C. Sections were washed twice for five minutes in PBS, followed by the addition of horseradish peroxidase conjugated anti-rabbit IgG (Amersham) at a dilution 1/200 in 4% BSA in PBS for 30 minutes at room temperature. After washing twice for five minutes in PBS, antibody location was determined with the addition of DAB chromogen (Dako, Glostrup, Denmark):3% H_2O_2 (130:1) for 10 to 15 minutes. Color development was stopped by washing in water. Sections were counterstained with Carazzi's hematoxylin (Bio-optica, Milano, Italy), dehydrated and mounted in Canada balsam (DPX, Poole, UK). As the negative control, nonspecific rabbit IgG was used in the place of the primary antibody.

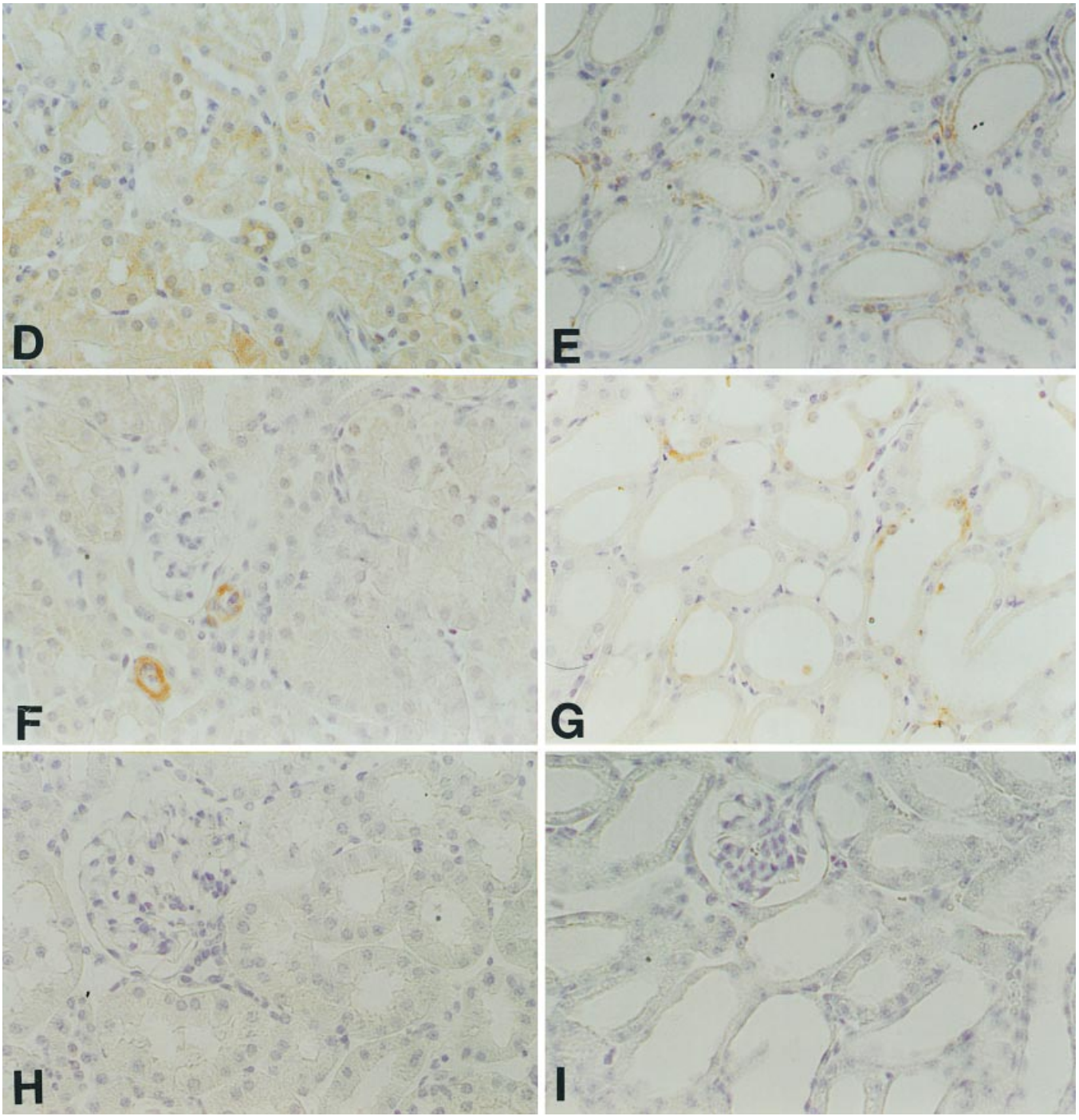

Fig. 3. (Continued).

Statistics

Results are expressed as mean ± SEM. Significance at the 95% level was established using one-way ANOVA and Student's *t*-test. The presence of significant differences between groups was examined by a post hoc test (Bonferroni's method) by means of the SigmaStat statistical software (Jandel, San Rafael, CA, USA).

RESULTS

Acute renal failure induced by folic acid is associated with changes in the expression of cell death genes

Folic acid nephropathy is a classical model of ARF with tubular injury [30]. Microscopic examination of kidney tissue from mice with ARF revealed the presence of tubular epithelial cells with pyknotic nuclei typical of

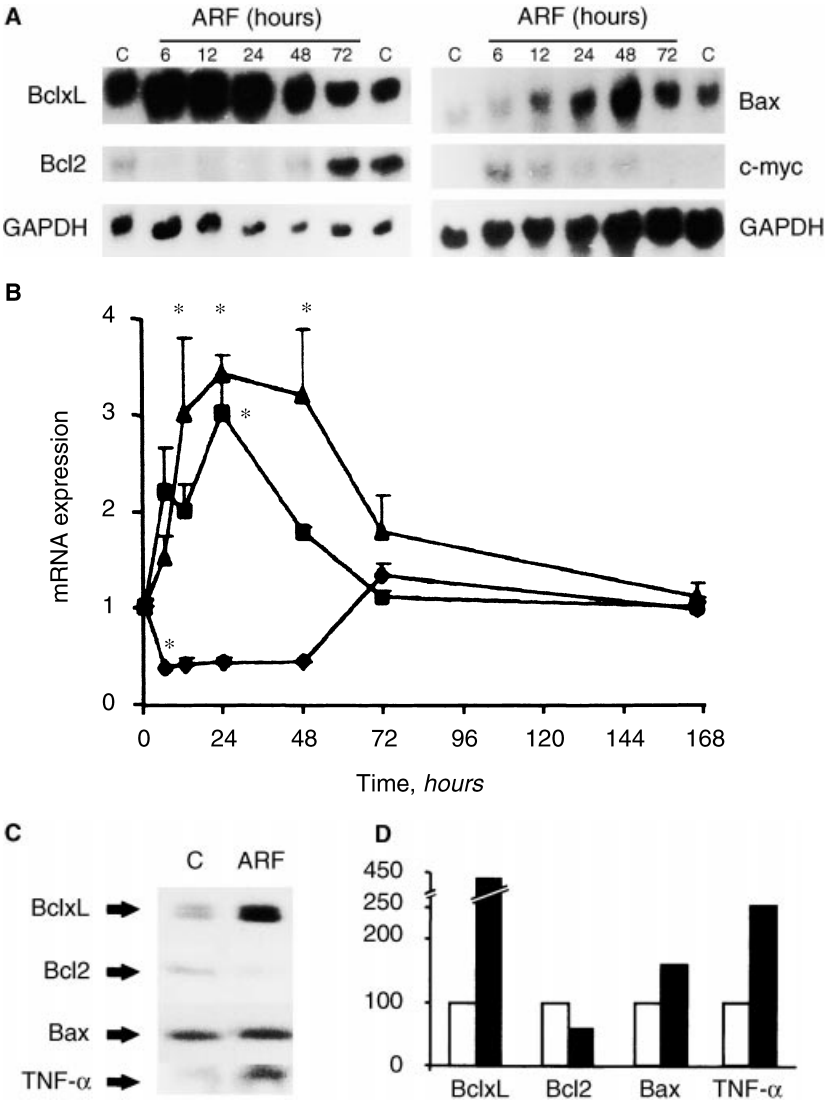

Fig. 2. Cell death genes in ARF induced by folic acid. (*A*) Northern blot. Gels were loaded with 30 μg of total RNA isolated from whole kidneys of mice that had been injected with folic acid or vehicle. Membranes were sequentially hybridized with probes for the indicated cell death genes. (*B*) Cell death gene expression changes during the first 48 hours of evolution of disease. Three different blots from different mice were analyzed by densitometry, and results expressed as arbitrary densitometry units normalized to GAPDH mRNA. Data are mean ± SEM, $*P < 0.05$ vs. control. Symbols are (◆) Bcl2; (■) BclxL; (▲) Bax. (*C*) Western blot (24 h). (*D*) Western blot densitometry in (□) control and (■) ARF mice. Mean of 2 mice.

apoptosis not present in control kidneys (Fig. 1A). In addition, the genomic DNA from kidneys harvested within 24 hours of folic acid administration showed internucleosomal fragmentation which was not present in control kidneys (Fig. 1B). Mean serum creatinine was fivefold increased in mice injected with folic acid with respect to vehicle-injected mice (1.5 ± 0.2 vs. 0.3 ± 0.01 mg/dL, $P < 0.05$).

To assess changes in mRNA transcript levels of various cell death genes, Northern blot analysis was performed on RNA isolated from murine kidneys at various times after the administration of folic acid or control vehicle. Six hours after administration of folic acid, there were elevations in mRNA levels encoding *c-myc*, *Bax*, and *Bclx*, while mRNA levels encoding *Bcl2* fell (Fig. 2A). The up-regulation of *bclx* expression was also observed with the use of a *BclxL*-specific probe.

mRNA encoding *c-myc* showed a peak 25-fold elevation at six hours, *Bax* showed a peak 3.6-fold elevation at 24 hours, and *BclxL* showed a peak threefold elevation at 24 hours (Fig. 2B). The mRNA encoding *Bcl2* decreased by 40% at six hours and remained depressed for 48 hours. The mRNA levels for all of these genes returned toward normal by 72 hours and remained normal at seven days. As peak changes in the renal expression of cell death genes occurred at 24 hours, Bcl2, BclxL and Bax proteins were assessed 24 hours after injection of folic acid. Western blot demonstrated that BclxL, but not BclxS was detectable in control and ARF kidneys (Fig. 2C). Bcl2 protein decreased 30%, BclxL increased fourfold and Bax increased slightly.

Protein immunohistochemistry revealed that the distribution of increased BclxL protein in ARF was patchy, with some tubules showing intense staining while some cells were negative (Fig. 3 B, C). By contrast, a diffuse decrement in Bcl2 staining was noted (Fig. 3E). Vascular

smooth muscle cells were the main site of Bax expression in the normal murine kidney (Fig. 3F). In ARF increased expression of Bax was patchy in whole tubuli and isolated tubular cells, including those detached into the tubular lumen (Fig. 3G). No significant staining was found in glomeruli with any of the antibodies.

TNF-α has also been shown to play a role in other models of acute renal failure [14, 15]. Interestingly, increased renal TNF-α expression was also noted 24 hours after disease induction in this model of ARF (twofold increase over control; Fig. 2C).

Death of cultured tubular cells induced by serum deprivation and TNF-α stimulation has features of apoptosis

A relative deficit of cytokines with survival factor activity has been reported in ARF [7–10], and we tested the effect of depriving cultured tubular epithelial cells of the survival factors present in serum. MCT cells grown in serum-free conditions accumulate more slowly than those grown in media supplemented with 10% FCS (cell number after 72 h in 10% FCS 2190 ± 54 vs. 116 ± 6 × 10^2 cells in serum-free media, $P < 0.0001$; Fig. 4A). While decreased cell proliferation in the absence of serum has been previously noted [31], increased cell death also contributed to lower cell numbers (Fig. 4B). After 72 hours in serum-free culture 35.9 ± 2% of the cells were dead, versus 9.6 ± 1% in the controls grown in the presence of 10% FCS ($P < 0.0001$). Dead cells had characteristic features of apoptotic cell death. Apoptotic bodies were observed among serum-deprived cells (Fig. 5A). Loss of cell contact is an early feature of apoptosis [32]. An increased number of detached cells with pyknotic nuclei were also noted in the supernatants of the MCT cells grown in serum-free conditions. While genomic DNA from the attached cells grown in serum-free conditions displayed internucleosomal DNA fragmentation, this pattern was much more evident in the detached cells (Fig. 5B). To further estimate the cell death attributable to apoptosis, Southern blot analysis was performed on the DNA obtained from detached cells floating in the culture supernatant with probe being total genomic DNA. The radioactivity bound to the low molecular weight DNA obtained from floating cells was counted and then normalized for the number of attached cells. Low molecular weight DNA isolated from supernatants after 72 hours of serum-free culture bound fourfold more counts than DNA isolated from the supernatants of the culture grown in the presence of 10% FCS.

In addition to growth factor deprivation, we wanted to examine whether factors known to induce apoptosis in other cells would induce apoptosis in MCT cells. One such factor, TNF-α, is increased in ARF induced by folic acid (above). Therefore, TNF-α at a final concentration of 30 ng/mL was added to the culture media of cells growing in the presence of 10% serum or cells that had been serum-deprived for 24 hours. TNF-α increased the rate of cell death in MCT cells more markedly in the serum-free cultures than in the serum containing cultures. Under serum-free growth conditions 80.1 ± 1.2% of the cells exposed to TNF-α were dead at 72 hours, compared to 43.8 ± 5% in the control cultures ($P < 0.01$) as assessed by trypan blue exclusion (Fig. 4). Consistent with this increased cell death, the total cell number at 72 hours was also decreased in TNF-α-treated cells (TNF-α 900 ± 63, serum-free control 6023 ± 214 cells/well, $P < 0.01$). The cytotoxic effect of TNF-α was less marked in the presence of serum (TNF-α 15.8 ± 0.9%, 10% FCS control 9.5 ± 1% dead cells at 72 h, $P < 0.01$). The cell death induced by TNF-α had characteristics of apoptosis. The number of cells floating in the supernatant of cultures to which TNF-α was added was threefold greater by 48 hours than the comparable control culture. Examination of these floating cells confirmed apoptotic changes including pyknotic nuclei (not shown) and internucleosomal DNA fragmentation (Fig. 5B).

Flow cytometry confirmed the presence of apoptotic hypodiploid cells (Ao peak) among those treated with serum deprivation or addition of TNF-α (Fig. 5C), the magnitude of cell death being similar to that obtained in trypan blue exclusion experiments (data not shown).

mRNA levels of apoptosis regulatory genes and their protein products in MCT cells change during serum deprivation and TNF-α stimulation

Serum deprivation of MCT cells modulated the expression of genes that regulate apoptosis. *Bax* mRNA expression was elevated 24 to 72 hours after withdrawal of serum (1.6 ± 0.15-fold at 48 h, peak 2.9-fold at 72 h), while *BclxL* and *Bcl2* message levels decreased (0.72 ± 0.06 and 0.62 ± 0.14 at 48 h; Fig. 6 A, B). These changes result in decreased *Bcl2*/*Bax* and *BclxL*/*Bax* ratios, which reflect a change in the balance between cell life- and death-promoting genes (*Bcl2*/*Bax* and *BclxL*/*Bax* mRNA ratios in cells deprived of serum for 48 hours were 39% and 44% those of cells grown in serum). Protein expression was studied by Western blot at 48 hours. Bax protein expression increased twofold, while that of BclxL decreased 45% and Bcl2 decreased 30% (Fig. 6C). The Bcl2/Bax and BclxL/Bax protein ratios in cells deprived of serum for 48 hours decreased (Fig. 6D).

Addition of TNF-α to serum-deprived cells resulted in further changes in the expression of apoptosis genes. The earliest change was a 3.5-fold increase in *c-myc* mRNA that was already evident at one hour (Fig. 7A). The mRNA encoding *Bcl2* reached a nadir at eight hours (45% of control), with almost total recovery by 48 hours while mRNA encoding *BclxL* decreased progressively with a nadir at 48 hours (60% of control) (Fig. 7 A, B). Western blot demonstrated that TNF-α did not induce

changes in Bax protein (Fig. 7 C, D) nor did it induce BclxS expression (not shown). TNF-α also did not appreciably change Bcl2 protein levels but it reduced BclxL protein 30% at 48 hours (Fig. 7 C, D).

BclxL overexpression protects from TNF-α induced apoptosis

A reduced BclxL/Bax ratio may contribute to the increased sensitivity of serum-deprived MCT cells to death induced by TNF-α. Supporting this hypothesis, overexpression of *BclxL* reduced the amount of apoptosis induced by TNF-α in serum-deprived cells by 71% when compared with control vector-expressing cells, as assessed by flow cytometry of DNA content (Fig. 8).

DISCUSSION

Cell death by apoptosis is known to occur in the kidney during ARF [1, 2], and we have confirmed its presence in ARF induced in mice by folic acid. Cell death results from the interplay and balance of survival and mortal signals [5]. From this perspective, an absolute or relative deficit in survival factors in damaged kidney facilitates renal cell death. Cell death is also modulated by a changing balance in protective and lethal intracellular proteins [19]. We now report changes in the expression of apoptosis regulatory proteins in an experimental model of ARF. Some features of this process in damaged kidney are reproducible in cultured tubular epithelial cells. As both decreased expression of cytokines with survival factor activity and the participation of cytokines that can promote cell death, such as TNF-α, have been reported in ARF [7–10, 14, 15], we subjected cultured tubular cells to deprivation of survival factors (serum deprivation) and/or the addition of a lethal factor (TNF-α). This microenvironment resulted in apoptotic cell death of tubular MCT cells and in changes in the expression of apoptosis regulatory genes.

The administration of folic acid induced ARF, increased renal levels of mRNA encoding c-myc and modulated the expression of genes encoding members of the Bcl2-like family of proteins in the kidney. Under similar conditions, levels of mRNA encoding *Bcl2* were decreased, while the message of its endogenous antagonist, *Bax*, increase. This resulted in a decreased *Bcl2/Bax* ratio that favored cell death [18]. By contrast, levels of mRNA encoding *BclxL* were elevated early in injury and were associated with a patchy increase in BclxL protein in tubules. These changes resemble those reported in a well-characterized model of epithelial cell apoptosis where involution of the mammary gland is associated with weaning. Apoptosis of mammary epithelial cells is also associated with an increased expression of *c-myc*, *Bax* and *Bcl2* and decreased *Bcl2* mRNA [33–35]. In vivo expression of *Bcl2*-related genes has also been studied

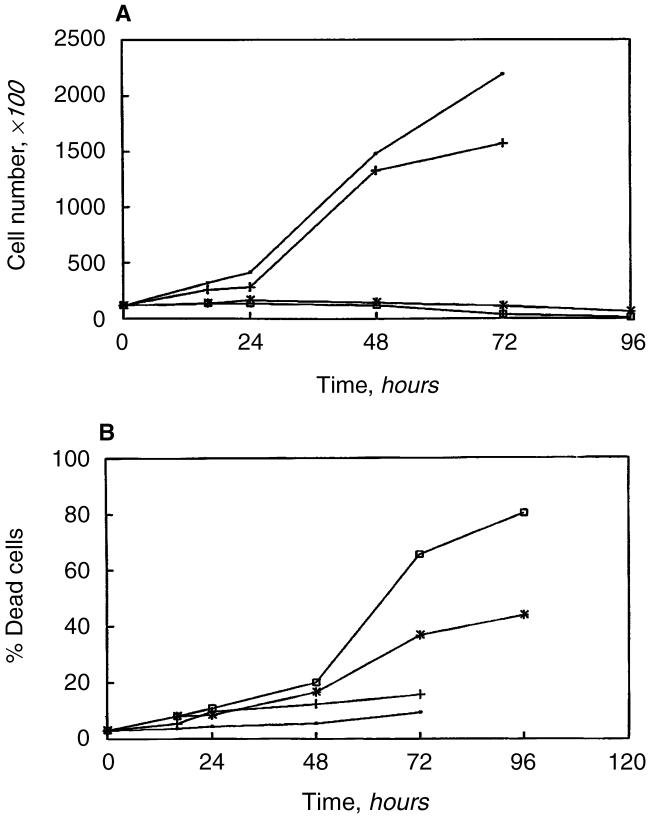

Fig. 4. Serum deprivation and addition of TNF-α are cytotoxic for MCT cells. (*A*) Both serum deprivation and addition of 30 ng/mL TNF-α resulted in MCT cell death, as assessed by trypan blue exclusion. The combination of the two stimuli increased cell death. (*B*) Increased cell death was associated with decreased cell accumulation. Ten thousand MCT cells were seeded in 24-well plates and grown overnight in 10% FCS DMEM. Then, the medium was changed to either 10% FCS DMEM (•), 10% FCS DMEM with 30 ng/mL TNF-α (+), or serum-free DMEM (*). After 24 hours in serum-free medium, 30 ng/mL TNF-α was added to some wells (□). Mean of quadruplicate wells (SEM < 5%).

extensively in the central nervous system. In these studies decreased levels of *Bcl2* and increased *Bax* mRNA is associated with apoptosis induced by ischemia or in amyotrophic lateral sclerosis [36, 37].

In ARF, however, there have been conflicting reports regarding the expression of mRNA encoding *Bcl2*. Increased, decreased or unchanged levels were found in ARF in rats given glycerol or $HgCl_2$, made ischemic, or following unilateral ureteral obstruction [38–40]. The reasons for such divergent findings are unclear. They may reflect different degrees or mechanisms of tissue injury or differences between species. In this regard, levels of renal Bcl2 were reported preliminarily to be low during obstructive renal damage [39] and obstruction of renal tubuli participates in the pathogenesis of folic acid nephropathy [23]. In folic acid nephropathy *Bcl2* expression decreased globally in tubular cells. However, it is conceivable that in other models of ARF or in other species, there may be a compensatory increase in Bcl2

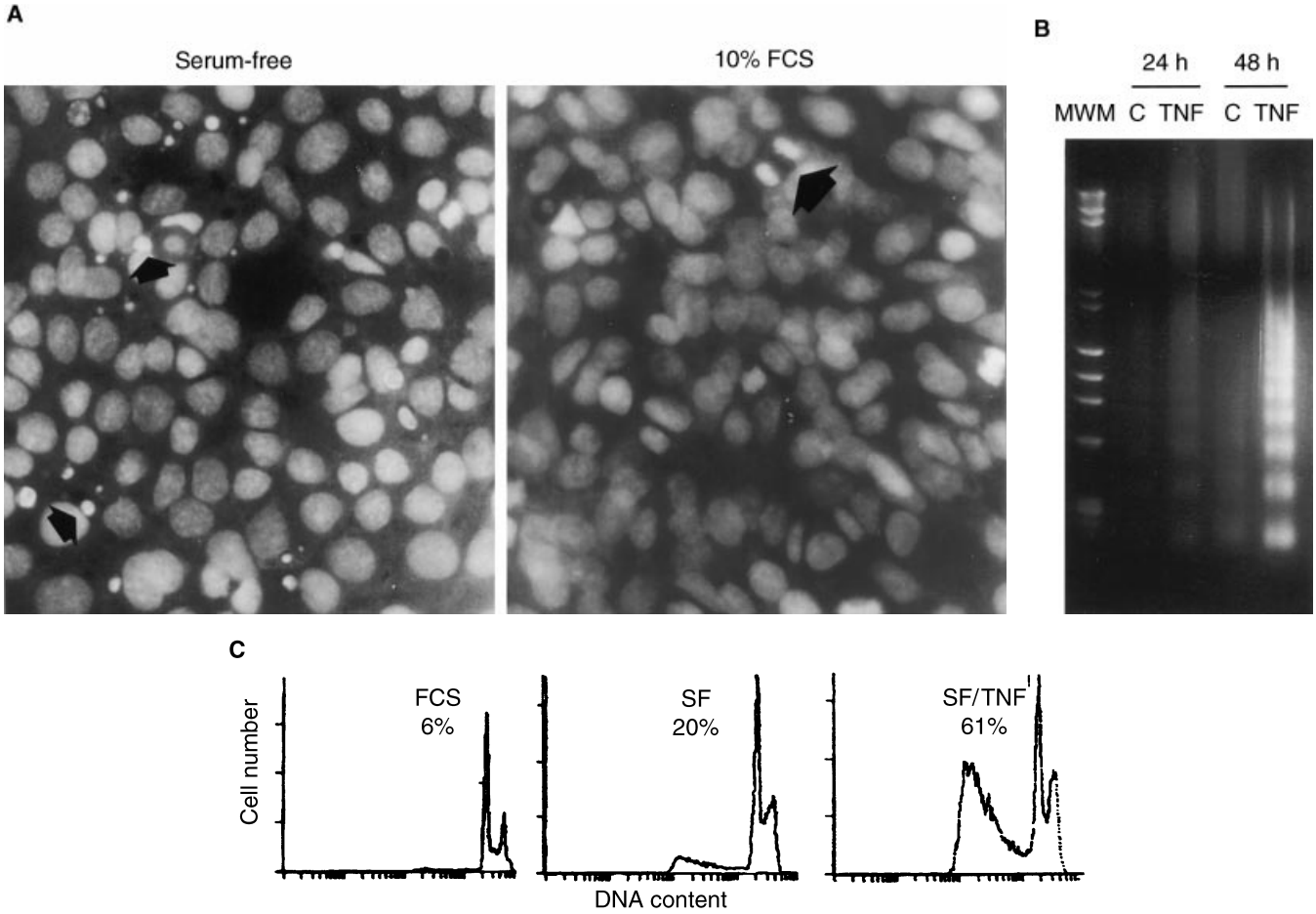

Fig. 5. Cell death induced by serum deprivation and TNF-α had features of apoptosis, such as the typical morphology, and internucleosomal DNA fragmentation. (*A*) Apoptotic bodies are observed in propidium iodide stained MCT cells grown in serum-free medium for 48 hours (arrows). By contrast, mitotic figures were prominent in cells grown in 10% FCS DMEM (arrows). (*B*) Internucleosomal DNA fragmentation of detached cells is already evident after culture for 24 hours in serum-free medium in the presence of 30 ng/mL TNF-α. After 48 hours, fragmented DNA is also present in cells cultured in control serum-free medium, but more prominent in cells cultured in the presence of TNF-α. (*C*) Flow cytometry of permeabilized, propidium iodide-stained control cells grown in 10% FCS, serum-depleted cells (SF) and serum-depleted cells treated for 48 hours with 30 ng/mL TNF-α. Note the apoptotic cells with hypodiploid DNA content.

in certain cells or tubules. Increased levels of mRNA encoding *Bax* during ARF has also been reported after renal ischemia in rats [40]. Immunohistochemistry revealed that in folic acid nephropathy increased Bax expression is not widespread among tubular cells, but rather, it is increased in individual tubules and tubular cells, probably those destined to die. There data are consistent with previous findings in the central nervous system and other systems [41, 42].

In cultured tubular MCT cells we identified a factor that may contribute to decreased expression of intracellular survival proteins and increased *Bax* expression in ARF. Survival factor deprivation by lowering levels of serum in culture media increased levels of *Bax* mRNA and protein, and decreased the *Bcl2*/*Bax* and *BclxL*/*Bax* ratios. This pattern of gene expression favors cell death [18, 43]. Increased *Bax* expression has been associated with apoptosis induced by withdrawal of survival factors in nonrenal cells, including epithelial cells [44]. In fact, evidence from neurons harvested from *Bax*-deficient mice suggests that Bax is required for apoptosis induced by deprivation of survival factors [45]. Deprivation of the survival factors present in serum also decreased *BclxL* mRNA and protein in cultured tubular cells. These findings are consistent with the patchy changes observed in vivo in the expression of *BclxL* and *Bax*. Indeed, the variability in expression of cytokines with survival activity in tubular cells, like the decrease in IGF-1 (with increased numbers of IGF-1 receptors) observed in folic acid nephropathy [7], suggests that tubular epithelium may compete for limiting amounts of survival factors by up-regulating their receptors, and some cells may even become deprived of them [5].

We therefore hypothesize that rate-limiting competition for survival factors contributes to the patchy increment in BclxL expression in ARF. Cells that do not

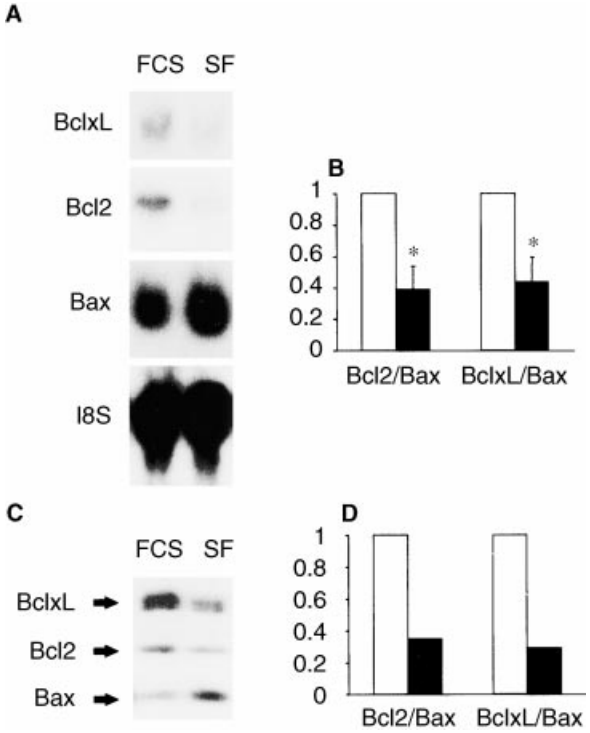

Fig. 6. Deprivation of the survival factors present in serum modulates the expression of cell death genes and their products. (*A*) Northern blot. Gels were loaded with 30 μg of total RNA obtained from MCT cells grown in either 10% FCS or in serum-free medium for 48 hr. Membranes were sequentially hybridized with Bcl2, BclxL, Bax and 18S. (*B*) Bcl2/Bax and BclxL/Bax mRNA ratios after 48 hours of serum deprivation (mean ± SEM of 3 independent experiments; $*P < 0.05$). (*C*) Western blot. Cells were cultured for 48 hours in the presence of 10% FCS or under serum-free conditions. (*D*) Western blot densitometry. Mean of 2 independent experiments: Bcl2/Bax and BclxL/Bax protein ratios after 48 hours of serum deprivation in (□) FCS or (■) serum-free conditions.

have access to survival factors down-regulate *BclxL*. In contrast, the response of cells to cytokines with survival factor activity is to increase expression of *BclxL* and be protected from cell death. This notion is consistent with the findings of increased levels of BclxL in surviving neurons within ischemic areas of the brain, while damaged neurons had decreased BclxL [42]. Increased expression of proteins that protect from apoptosis by surviving tubular cells may promote tubular regeneration during the recovery phase of ARF. Indeed, renal regeneration in folic acid nephropathy may be exuberant and lead to increased kidney weight [46]. The degree of protection afforded by BclxL depends on the balance of moieties that favor apoptosis. BclxS may counterbalance the protective effect of an excess amount of BclxL expression [35]. However, expression of BclxS could not be detected in either our in vitro or in vivo systems.

Serum-deprived tubular cells showed a pro-death pattern of cell death protein expression (decreased Bcl2/Bax and BclxL/Bax ratios) and were, indeed, more susceptible to apoptosis induced by TNF-α, a cytokine that promotes tubular cell death and is increased in ARF induced by folic acid. In this regard, enforced BclxL overexpression protected from TNF-α-induced apoptosis. In addition, TNF-α progressively lowered the expression of mRNA encoding *BclxL*. These findings are consistent with previous reports of TNF-α being able to down-regulate the expression of *Bclx* in nonrenal cells [47]. The time course of the changes in *Bclx* expression is consistent with a role in the delayed effect of TNF-α on cell survival. By contrast, TNF-α transiently decreased *Bcl2* mRNA while Bcl2 protein did not change. This dissociation between *Bcl2* mRNA and protein expression has been described in other cell systems, and may be related to the long half-life of the Bcl2 protein [48, 49] or to differential regulation of mRNA and protein half-lives [50, 51]. The transient nature of the decrement in levels of mRNA encoding *Bcl2* may prevent Bcl2 protein from falling in MCT cells.

The Bcl2 family of proteins includes other members that protect from (Bclw, A1) or promote (Bad, Bak, BH3-only proteins) cell death [19]. At least some of them are expressed by tubular epithelium and could play a role in ARF. In addition, Bcl2 and BclxL can prevent cell death with features of necrosis [20], and it is conceivable that their role during ARF extends beyond apoptosis to influence other forms of cell death.

Increased *c-myc* mRNA had been previously reported in the early stages of folic acid nephropathy and other models of ARF [52, 53], where it was thought to play a role in compensatory cell proliferation. However, recent data suggest that cell proliferation and apoptosis may share a common initial pathway, and that availability of additional external survival factors and the expression of cell death protection genes may better determine cell fate. Overexpression of *c-myc* leads to cell proliferation in the presence of serum or high levels of intracellular protective proteins, but cells with deregulated *c-myc* expression undergo apoptosis if survival factors are withdrawn or Bcl2 levels are low [17, 54]. In a similar manner, we propose that increased levels of *c-myc* in ARF may regulate cell number depending on the local availability of survival factors. The increased sensitivity to TNF-α-induced cell death of serum-deprived tubular cells may be related to the high *c-myc* expression induced by TNF-α. Induction of *c-myc* expression had previously been associated with increased TNF-α cytotoxicity [55] and survival factors also prevent TNF-α-induced cell death in oligodendrocyte cultures [4].

Taken together, the in vitro data demonstrating an influence of extracellular factors with survival and lethal activity on the expression of apoptosis regulatory proteins in tubular cells, the in vivo data of similar changes in ARF, and previous evidence for absolute or relative deficit of cytokines with survival factor activity and in-

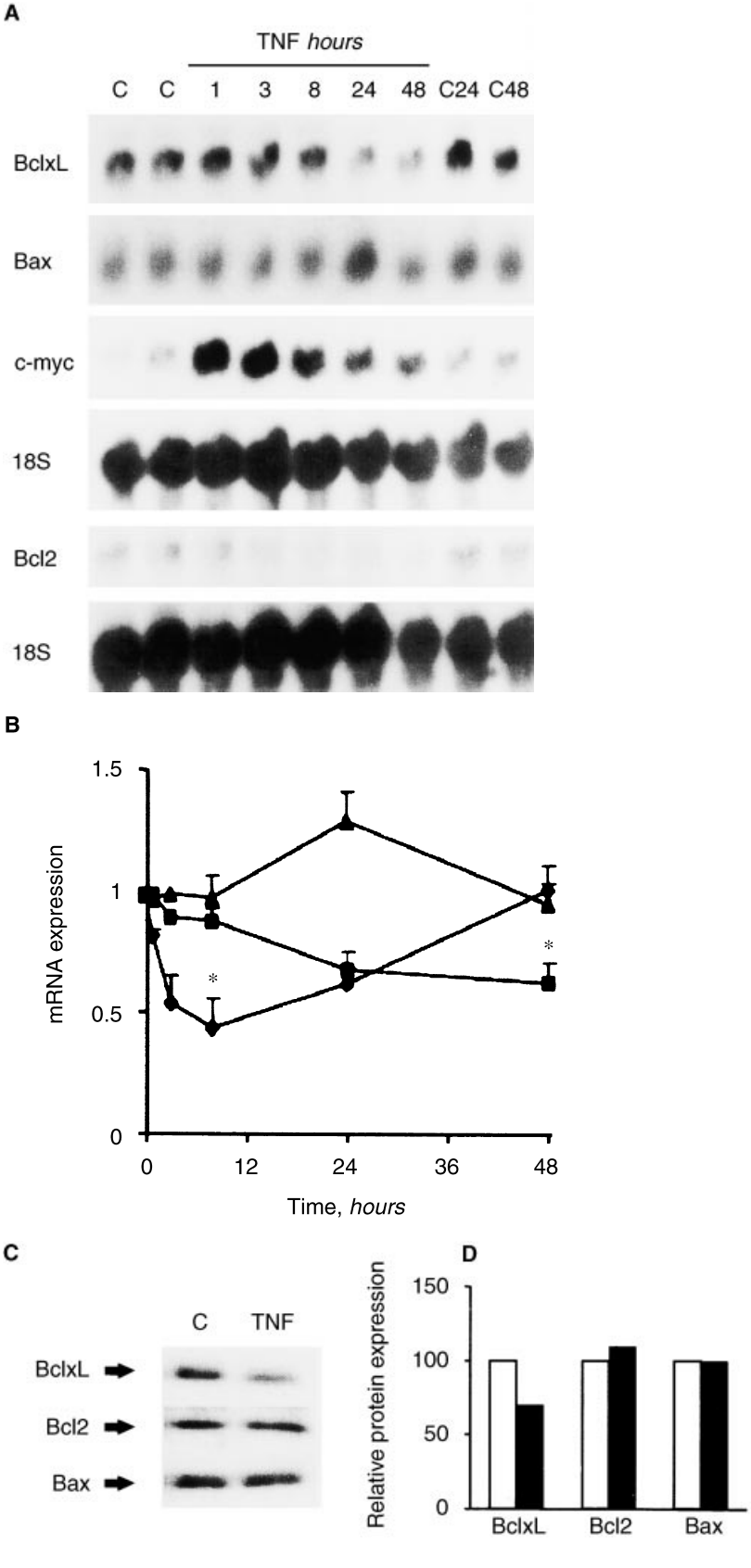

Fig. 7. TNF-α modulates the expression of cell death genes and their products. (*A*) Northern blot. Gels were loaded with 30 μg of total RNA obtained from MCT cells grown in serum-free medium for 24 hours, before adding either control media or 30 ng/mL TNF-α for 1 to 48 hours. Membranes were sequentially hybridized with *Bcl2*, *Bclx*, *Bax*, c-*myc*, and 18S. (*B*) Densitometry of the previous membranes. RNA expression was normalized for 18S, and results are expressed in arbitrary densitometry units in relation to controls cultured for similar time periods (▲-*Bax*; ●-*Bcl2*; ■-*Bclx*). Mean ± SEM of three independent experiments. $^*P < 0.05$ versus control. (*C*) Western blot of MCT cells cultured for 48 hours in the presence or absence of 30 ng/mL TNF-α. (*D*) Western blot densitometry (□-Control; ■-TNF-α). Mean of 2 independent experiments.

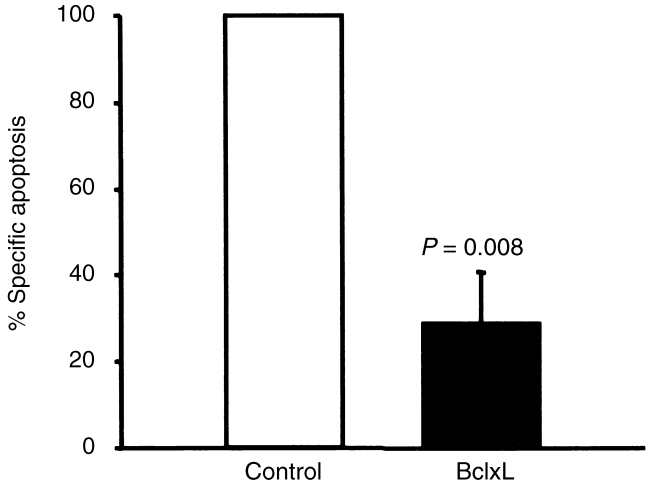

Fig. 8. BclxL protects from TNF-α-induced apoptosis. Apoptosis induced at 48 hours by 30 ng/mL TNF-α in serum-deprived cells overexpressing *BclxL* or control cells was quantified by flow cytometry. Mean ± SEM of 5 independent experiments.

creased release of lethal factors during ARF, suggest that apoptosis regulatory proteins like c-Myc, Bcl2, Bax and Bclx may have a role in the regulation of tubular cell death during ARF. The precise role of each of these proteins in renal disease remains to be clarified. The better understanding of the role and regulation of apoptotic cell death in acute renal injury may provide new insights into the pathogenesis of ARF and provide the basis for new therapeutic strategies. Understanding the regulation of tubular cell apoptosis may be also relevant to the pathogenesis of tubular atrophy in chronic renal disease [3]. Tubular cell apoptosis has been observed in this setting and lethal cytokines, such as TNF-α and Fas ligand, may leak from the inflamed glomeruli and reach the tubular epithelium or may be released from infiltrating lymphocytes or interstitial fibroblasts.

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REFERENCES

1. Schumer M, Colombel M, Sawczuk I, Gobe G, Connor J, O'toole K, Olsson C, Wise G, Buttyan R: Morphologic, biochemical and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischemia. *Am J Pathol* 140:831–838, 1992
2. Olsen S, Burdick J, Keown P, Wallace A, Racussen L, Solez K: Primary acute renal failure ("acute tubular necrosis") in the transplanted kidney: Morphology and pathogenesis. *Medicine* 68:173–187, 1989
3. Gobe G, Axelsen R, Searle J: Cellular events in experimental unilateral ischemic renal atrophy and in regeneration after contralateral nephrectomy. *Lab Invest* 63:770–779, 1990
4. Louis J, Magal E, Takayama S, Varon S: CNTF protection of oligodendrocytes against natural and tumor necrosis factor-induced death. *Science* 259:689–692, 1993
5. Raff M: Social controls on cell survival and cell death. *Nature* 356:397–400, 1992
6. Barres B, Hart I, Coles H, Burne J, Voyvodic J, Richardson W, Raff M: Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70:31–46, 1992
7. Hise M, Li L, Mantzouris N, Rohan R: Differential mRNA expression of insulin-like growth factor system during renal injury and hypertrophy. *Am J Physiol* 269:F817–F824, 1995
8. Safirstein R, Zelent A, Price P: Reduced renal pre-pro-epidermal growth factor mRNA and decreased EGF excretion in ARF. *Kidney Int* 36:810–815, 1989
9. Verstrepen W, Nouwen E, Yue X, De Broe M: Altered growth factor expression during proximal tubular necrosis and regeneration. *Kidney Int* 43:1267–1279, 1993
10. Tsao T, Wang J, Fervenza F, Vu T, Jin I, Hoffman A, Rabkin R: Renal growth hormone–insulin-like growth factor-I system in acute renal failure. *Kidney Int* 47:1658–1668, 1995
11. Kennedy W, Buttyan R, Garcia-Montes E, D'Agati V, Olsson C, Sawczuk I: Epidermal growth factor suppresses renal tubular apoptosis following ureteral obstruction. *Urology* 49:973–980, 1997
12. Chevalier R, Goyal S, Wolstenholme J, Thornhill B: Obstructive nephropathy in the neonatal rat is attenuated by epidermal growth factor. *Kidney Int* 54:38–47, 1998
13. Ortiz A, Lorz C, Gonzalez-Cuadrado S, Garcia del Moral R, O'Valle F, Egido J: Cytokines and Fas regulate apoptosis in murine renal interstitial fibroblasts. *J Am Soc Nephrol* 8:1845–1854, 1997
14. Gresser J, Woodrow D, Moss J, Maury C, Tavernier J, Fiers W: Toxic effects of recombinant tumor necrosis factor in suckling mice. Comparisons with interferon α/β. *Am J Pathol* 128:13–18, 1987
15. Shulman L, Yuhas Y, Frolkis I, Gavendo S, Knecht A, Eliahou H: Glycerol-induced ARF in rats is mediated by tumor necrosis factor alpha. *Kidney Int* 43:1397–1401, 1993
16. Boise L, Gonzalez-Garcia M, Postema C, Ding L, Lindsten T, Turka L, Mao X, Nunez G, Thompson C: bcl-x, a bcl-2-related gene that functions as a dominant regulator of cell death. *Cell* 74:597–608, 1993
17. Evan G, Wyllie A, Gilbert C, Littlewood T, Land H, Brooks M, Waters C, Penn L, Hancock D: Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69:119–128, 1992
18. Oltvai Z, Milliman C, Korsmeyer S: Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74:609–619, 1993
19. Reed JC: Bcl-2 family proteins. *Oncogene* 17:3225–3236, 1998
20. Kane D, Sarafian T, Anton R, Hahn H, Gralla E, Valentine J, Ord T, Bredesen D: Bcl2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* 262:1274–1277, 1993
21. Martinou J, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C: Overexpression of Bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* 13:1017–1030, 1994
22. Vaux DL: Toward an understanding of the molecular mechanisms of physiological cell death. *Proc Natl Acad Sci USA* 90:786–789, 1993
23. Fink M, Henry M, Tange J: Experimental folic acid nephropathy. *Pathology* 19:143–149, 1987
24. Haverty TP, Kelly CJ, Hines WH, Amenta PS, Watanabe M, Harper RA, Kefalides NA, Neilson EG: Characterization of a renal tubular epithelial cell line which secretes the autologous target antigen of autoimmune experimental interstitial nephritis. *J Cell Biol* 107:1359–1368, 1988
25. Kurauchi O, Lahuna O, Darbouy M, Aggerbeck M, Chobert M, Laperche Y: Organization of the 5′ end of the rat gamma-

glutamyl transpeptidase gene: Structure of a promoter active in the kidney. *Biochemistry* 30:1618–1623, 1991
26. González-Cuadrado S, López-Armada M, Gómez-Guerrero C, Subirá D, Ortiz-González A, Neilson E, Egido J, Ortiz A: Anti-Fas antibodies induce cytolysis and apoptosis in cultured human mesangial cells. *Kidney Int* 49:1064–1070, 1996
27. González-Cuadrado S, Lorz C, García del Moral R, O'Valle F, Alonso C, Ramiro F, Ortiz-González A, Egido J, Ortiz A: Agonistic anti-Fas antibodies induce glomerular cell apoptosis in mice in vivo. *Kidney Int* 51:1739–1746, 1997
28. Ortiz-Arduan A, Danoff T, Kalluri R, González-Cuadrado S, Karp S, Elkon K, Egido J, Neilson E: Regulation of Fas and Fas ligand expression in cultured murine renal cells and in the kidney during endotoxemia. *Am J Physiol* 241:F1193–F1201, 1996
29. Ortiz A, Ziyadeh F, Neilson E: Expression of apoptosis-regulatory genes in renal proximal tubular epithelial cells exposed to high ambient glucose and in diabetic kidneys. *J Invest Med* 45:50–56, 1997
30. Klingler E, Evan A, Anderson R: Folic acid-induced renal injury and repair. *Arch Pathol Lab Med* 104:87–93, 1980
31. Ziyadeh F, Snipes E, Watanabe M, Alvarez R, Goldfarb S, Haverty T: High glucose induces cell hypertrophy and stimulates collagen gene transcription in proximal tubule. *Am J Physiol* 259:F704–F714, 1990
32. Arends M, Wyllie A: Apoptosis: Mechanisms and role in pathology. *Int Rev Exp Pathol* 32:223–253, 1991
33. Strange R, Li F, Saurer S, Burkhardt A, Friis R: Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development* 115:49–58, 1992
34. Merio G, Cella N, Hynes E: Apoptosis is accompanied by changes in Bcl-2 and Bax expression, induced by loss of attachment, and inhibited by specific extracellular matrix proteins in mammary epithelial cells. *Cell Growth Differ* 8:251–260, 1997
35. Heermeier K, Benedict M, Li M, Furth P, Nunez G, Hennighausen L: Bax and Bcl-xs are induced at the onset of apoptosis in involuting mammary epithelial cells. *Mech Dev* 56:197–207, 1996
36. Gillardon F, Lenz C, Waschke K, Krajewski S, Reed J, Zimmermann M, Kuschinsky W: Altered expression of Bcl-2, Bcl-X, Bax, and c-Fos colocalizes with DNA fragmentation and ischemic cell damage following middle cerebral artery occlusion in rats. *Brain Res Mol Brain Res* 40:254–260, 1996
37. Mu X, He J, Anderson D, Trojanowski J, Springer J: Altered expression of bcl-2 and bax mRNA in amyotrophic lateral sclerosis spinal card motor neurons. *Ann Neurol* 40:379–386, 1996
38. Nath K, Croatt A, Likely S, Behrens T, Warden D: Renal oxidant injury and oxidant response induced by mercury. *Kidney Int* 50:1032–1043, 1996
39. Chevalier R: Growth factors and apoptosis in neonatal ureteral obstruction. *J Am Soc Nephrol* 7:1098–1105, 1996
40. Basile D, Liapis H, Hammerman M: Expression of bcl-2 and bax in regenerating rat renal tubules following ischemic injury. *Am J Physiol* 272:F640–F647, 1997
41. Boersma A, Nooter K, Burger H, Kortland C, Stoter G: Bax upregulation is an early event in cisplatin-induced apoptosis in human testicular germ-cell tumor cell line NT2, as quantitated by flow cytometry. *Cytometry* 27:275–282, 1997
42. Isenmann S, Stoll G, Schroeter M, Krajewski S, Reed J, Bahr M: Differential regulation of Bax, Bcl-2, and Bcl-X proteins in focal cortical ischemia in the rat. *Brain Pathol* 8:49–62, 1998
43. Simonian P, Grillot D, Merino R, Nunez G: Bax can antagonize Bcl-XL during etoposide and cisplatin-induced cell death independently of its heterodimerization with Bcl-XL. *J Biol Chem* 271: 22764–22772, 1996
44. Hass R, Busche R, Luciano L, Reale E, Engelhardt W: Lack of butyrate is associated with induction of Bax and subsequent apoptosis in the proximal colon of guinea pig. *Gastroenterology* 112:875–888, 1997
45. Deckwerth T, Elliott J, Knudson C, Johnson EJ, Snider W, Korsmeyer S: BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron* 17:401–411, 1996
46. Threlfall G, Taylor D, Buck A: The effect of folic acid on growth and deoxyribonucleic acid synthesis in the rat kidney. *Lab Invest* 15:1477–1485, 1996
47. Lin R, Hwang Y, Yang B, Lin C: TNF receptor-2-triggered apoptosis is associated with the down-regulation of Bcl-xL on activated T cells and can be prevented by CD28 costimulation. *J Immunol* 158:598–603, 1997
48. Kitada S, Krajewski S, Miyashita T, Krajewska M, Reed J: Gamma-irradiation induces upregulation of Bax protein and apoptosis in radiosensitive cells in vivo. *Oncogene* 12:187–192, 1996
49. Delia D, Aiello A, Formelli F, Fontanella E, Costa A, Miyashita T, Reed J, Pierotti M: Regulation of apoptosis induced by the retinoid N-(4-hydroxyphenyl) retinamide and effect of deregulated bcl-2. *Blood* 85:359–367, 1995
50. Blagosklonny M, Alvarez M, Fojo A, Neckers L: bcl-2 protein downregulation is not required for differentiation of multidrug resistant HL60 leukemia cells. *Leuk Res* 20:101–107, 1996
51. Hu Z, Minden M, McCulloch E: Post-transcriptional regulation of bcl-2 in acute myeloblastic leukemia: significance for response to chemotherapy. *Leukemia* 10:410–416, 1996
52. Cowley B, Chadwick L, Grantham J, Calvet J: Sequential protooncogene expression in regenerating kidney following acute renal injury. *J Biol Chem* 264:8389–8393, 1989
53. Sawczuk I, Hoke G, Olsson C: Gene expression in response to acute unilateral ureteral obstruction. *Kidney Int* 35:1315–1319, 1989
54. Bissonnette R, Echeverri F, Mahboubi A, Green D: Apoptotic cell death induced by c-myc is inhibited by bcl-2. *Nature* 359:552–554, 1992
55. Klefstrom J, Vastrik I, Saksela E, Valle J, Eilers M, Alitalo K: c-Myc induces cellular susceptibility to the cytotoxic action of TNF-alpha. *EMBO J* 13:5442–5450, 1994